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(54) Title: TISSUE PLASMINOGEN ACTIVATOR

(57) Abstract

Tissue plasminogen activator (tPA) derived from the non-cancerous mammalian cell line JMI-229 is found to have significantly greater binding affinity to human fibrin clots than currently available therapeutic-grade tPA, which is based on human tPA, thus exhibiting improved properties for potential therapeutic use.

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Title: Tissue Plasminogen Activator

Field of Invention

This invention concerns tissue plasminogen activator and the use thereof as a thrombolytic agent.

Background to the Invention

One of the major causes of death in the Western World is myocardial infarction (MI), which is often caused by the obstruction of a coronary artery by a fibrin clot, or thrombus. Over recent years much attention has been paid to developing therapeutic treatments for dissolution of these blood clots, thereby recanalising the blood vessel and permitting the resumption of blood flow to the cardiac tissue.

The natural processes of clot formation and subsequent lysis involve the interaction and regulation of a large number of components. The bulk of the clot is composed of fibrin which itself is derived from circulating fibrinogen by the action of the enzyme thrombin. Subsequent clot lysis is heavily regulated in order to prevent premature dissolution of haemostatic plugs and inhibition of wound healing. The enzyme directly responsible for clot lysis is plasmin. Any free plasmin in the circulation is very rapidly inhibited by the action of alpha $_2$-antiplasmin. The circulating form of plasmin is the inactive zymogen,

plasminogen. This is activated to plasmin only on the clot surface by the action of tissue plasminogen activator (tPA). Activation of plasminogen by tPA only occurs when tPA is first bound to fibrin (Holylaerts, et al, J. Biol. Chem. (1982) 257: 2912-2919). The activity of tPA in plasma is further diminished by the presence there of a fast-acting tPA inhibitor - PAI-I (Wiman et al, Scan. J. Clin. Lab. Invest. (1985) 45 (Suppl. 1A) 43-47).

It is against this physiological background that efforts have been made to develop thrombolytic agents for therapeutic use against conditions which derive from obstruction of blood vessel by fibrin clots. These include myocardial infarction, pulmonary embolism, deep-vein thrombosis and stroke.

CURRENT THERAPEUTIC AGENTS:

Streptokinase

Streptokinase is a protein produced by certain strains of beta-haemolytic streptococci. It has no intrinsic enzymic activity, but forms a 1:1 stoichiometric complex with plasminogen which then undergoes a transition and exposes an active site in the modified plasminogen. The complex then becomes a potent plasminogen activator.

This agent has been the subject of several clinical trials (for review see Ward Kennedy, J. Am. Coll. Cardiol. (1987) 10(5) 28B-32B). It has been clearly shown that intracoronary or intravenous streptokinase therapy, when initiated within the first six hours of acute MI, reduces mortality.

Despite this clear clinical outcome, there is a significant problem associated with streptokinase therapy. This derives from the fact that the agent has no affinity for fibrin clots, and so activates plasminogen throughout the bloodstream. This leads to the so-called systemic fibrinolytic state where the level of circulating plasmin saturates the levels of its natural inhibitor alpha $_2$-antiplasmin. Circulating plasmin then proteolytically degrades many other components of the fibrinolytic system, especially fibrinogen.

APSAC

APSAC, or Anisoylated Plasminogen Streptokinase Activator Complex, is an inactive form of the plasminogen-streptokinase complex. Following injection, the complex is slowly deacylated, generating active complex over a prolonged time period (Ferres, et al, Drugs (1987) 33 (Suppl. 3) 80-82). The aim of this is to permit rapid administration of the agent over 5 minutes, rather than the prolonged infusions necessary for both streptokinase and tPA (see below).

Although preliminary clinical results (AIMS Trial Study Group, Lancet (1988) 8585 545-549) are encouraging with respect to mortality reduction, clinical problems with lack of clot selectivity persist (Conard, et al, Fibrinolysis 2 (Suppl. 1) 81 (1988)).

tPA

As a consequence of its fibrin specificity, a considerable amount of effort has been made to develop tPA as a thrombolytic agent. The gene for human tPA, isolated from

a Bowes melanoma cell line, has been cloned and expressed in chinese hamster ovary (CHO) cells and the resultant recombinant tPA (r tPA) used clinically. The efficacy of this agent in clinical trials has been reviewed (de Bono, J. Am. Coll. Cardiol. (1987) 10(5) 75B-78B: Sobel, J. Am. Coll. Cardiol. (1987) 10(5) 40B-44B). Coronary thrombolysis is achieved with a much reduced incidence of fibrinogenolysis when compared to streptokinase therapy.

However, tPA has a very short half-life *in vivo* of 3-6 minutes (Krause, Fibrinolysis (1988) 2 133-142) which has necessitated the administration of the agent at very high levels as an infusion over 6 hours. Under these conditions, where the plasma level of tPA is raised about 1000-fold over the physiological level, the clot specificity of the activation of plasminogen by tPA *in vivo* is relative and not absolute (Collen, Schweiz. Med. Wschr. (1987) 117(46) 1791-1798) and hence systemic activation of the fibrinolytic system occurs to a variable degree (Collen et al, Circulation (1986) 73 511-517).

THE SEARCH FOR IMPOROVED THROMBOLYTIC AGENTS

It was this realisation of the limitations of tPA under currently-used therapeutic regimes which has fuelled interest in 'second generation' thrombolytics which share the advantages but have fewer of the drawbacks than currently-available tPA. Numerous approaches are being utilised, eg site-directed mutagenesis, protein engineering and modification of carbohydrate moieties (for review see Pannekoek et al, Fibrinolysis (1988) 2 123-132, plus 97 refs. therein).

Several authors have detailed the aims of this work:

- 'Mutants of tPA, which have a higher fibrin-affinity, a longer in vivo half-life, or an improved fibrin-specificity may constitute potentially useful thrombolytic agents' - (Lijnen & Collen, Biotechnology in Clinical Medicine (1987), Albertini et al, editors, Raven Press, pp57-63).

- 'Mutants and hybrids of these molecules are being constructed and may further improve their fibrin specificity and therapeutic potential'. (Verstraete, J. Am. Coll. Cardiol. (1987) 10(5) 4B-10B).

- 'Improved molecules with respect to half-life, fibrin-binding and catalytic activity may be derived ..... with resulting beneficial therapeutic efficacy and for lower side-effect profiles'. (Harris, Protein Engineering (1987) 1(6) 449-458).

- 'It is becoming apparent that the fibrin specificity of these agents is not as pronounced in humans as was anticipated from several animal models. Therefore, the quest for thrombolytic agents with better fibrin selectivity continues.' (Collen, J. Am. Coll. Cardiol. (1987) 10(5) 11B-15B).

Summary of the Invention

The present invention is based on the unexpected discovery that a tissue plasminogen activator produced from a rat

liver-derived non-cancerous mammalian cell-line known as JMI-229 and deposited in the European Collection of Animal Cell Cultures at the PHLS, Centre for Applied Microbiology and Research, Porton Down, England under the accession number 89050502 exhibits (i) significantly greater binding affinity to human fibrin clots, (ii) altered pharmacokinetics in rabbits exhibiting an extended elimination phase, and (iii) increased efficacy at low doses in a rabbit arterio-venous shunt model than does the current therapeutic-grade tPA (Actilyse, Dr Karl Thomae, GmbH, FRG.)

Thus, in one aspect the present invention provides a pharmaceutical preparation comprising tPA derived from the non-cancerous mammalian cell line JMI-229.

The gene for tPA from this cell line may be cloned and expressed in other cell lines, particularly non-cancerous cell lines.

The invention thus also provides a pharmaceutical preparation comprising tPA derived from the gene for tPA in the cell line JMI-229, cloned and expressed in any other cell line.

It is believed tPA derived from other non-cancerous rat cell lines will have smilar therapeutically advantageous properties.

The amino acid sequence of JMI-229 tPA differs to that previously described for rat tPA, having serine as the N-terminal amino acid and glutamic acid at position 348 counted from the N-terminal end of the molecule.

The present invention thus also provides a pharmaceutical preparation comprising tPA derived from any rat cell line of non-cancerous origin, the tPA having serine as the N-terminal amino acid and glutamic acid at position 348 counted from the N-terminal end of the molecule.

tPA from a rat cell line may be cloned and expressed in other cell lines, particularly non-cancerous cell lines for production of material suitable and acceptable for use as a therapeutic agent.

The invention also includes within its scope a pharmaceutical preparation comprising tPA derived from the gene for rat tPA, cloned and expressed in any cell line, the tPA having serine as the N-terminal amino acid and glutamic acid at position 348 counted from the N-terminal end of the molecule.

In another aspect, the invention covers a pharmaceutical preparation comprising tPA having an amino acid sequence substantially identical to that for rat tPA derived from rat cells of non-cancerous origin, the tPA having serine as the N-terminal amino acid and glutamic acid at position 348 counted form the N-terminal end of the molecule.

The invention also includes within its scope a method of treating conditions which derive from obstruction of blood vessels of fibrin clots, comprising administering an effective amount of a preparation in accordance with the invention.

Previous work with rat tPA (Strickland D K et al, (1983) Biochemistry 22 4444-4449; Waller E K (1984) PhD thesis, Rockefellar Univ. NY) has used tPA derived from

adenocarcinoma cells and has concentrated on the biochemical characterisation of the protein, rather than its potential therapeutic use. Thus, fibrin-binding and efficacy in clot lysis were not examined.

A recent US patent (4,661,453) describes an elegant method for the production of tPA over prolonged periods from rat adenocarcinoma cells. These authors, however, indicate that the activity of their tPA was completely quenched by antiserum to Bowes melanoma tPA, and therefore concluded that tPA from rat adenocarcinoma cells was identical to Bowes melanoma tPA. These authors therefore failed to discover the potential therapeutic benefit of rat tPA over the tPA derived from Bowes melanoma (either directly or by cloning). Further, we must conclude that their experiments were not done in a manner which allowed the immunological differences between Bowes melanoma and rat adenocarcinoma cell tPA species to be apparent.

Improved affinity for fibrin clots, as demonstrated by tPA from JMI-229 cells, is one of the key criteria being sought for 'second generation' tPA molecules. (For references see above.) The consequences of increased affinity for fibrin clots go far beyond minimisation of systemic activation of the fibrinolytic system.

Enhanced fibrin binding would lead to each of the following:

- greater fibrin specificity leading to reduced systemic activation of the fibrinolytic system.

- greater apparent fibrin enhancement of plasminogen activation.

- more rapid clot dissolution.

- greater protection from inhibitors caused by rapid removal of the agent from plasma and localisation on fibrin clots.

In turn, the improved 'targeting' of tPA from JMI-229 to fibrin could permit lowering of the current dose requirements as evident in the Examples below. This would lead to a further reduction in side-effects and a significant improvement in the current cost of treatment.

Identification of Cell-Line JMI-229

In order to confirm the identify of cell-line JMI-229, DNA was extracted and subjected to fingerprint analysis using the mini-satellite probe 33.6. A well-characterised rat-liver cell line, RLC (deposited with the European Collection of Animal Cell Cultures, Porton Down, England under the accession number 86070103) was analysed in parallel and the results presented in Figure 1. Identical banding patterns are evident, indicating complete identity of the DNA of the two cell lines. For reference, DNA fingerprint analysis of the human cell line MRC-5 (deposited with the European Collection of Animal Cell Cultures, Porton Down, England under the accession number 85020201) resulted in a completely different banding pattern.

The Nature of JMI-229 tPA

The differences between the tPA molecule from cell-line JMI-229 and that derived form human Bowes melanoma, either

directly or by cloning and expression in other cells (rtPA), have been examined.

The results detailed below show clear differences between the tPA derived from cell-line JMI-229 and that isolated from Bowes melanoma cell lines and presently used as a thrombolytic agent. In particular, the tPA from JMI-229 showed enhanced fibrin binding activity and an exceptional affinity for fibrin clots.

(i) Molecular Weight

Both the one-chain and two-chain forms of JMI-229 and Actilyse have been subjected to polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS). Vertical resolving gels (0.75mm thickness) containing 12.5% (w/v) polyacrylamide were cast using the Laemmli buffer system (Laemmli, UK, Nature 227: 680-685, 1970). Between 1-3 ug of protein was loaded onto a 3% (w/v) polyacrylamide stacking gel. The gel was resolved under conditions of constant current, stained with Coomasie Blue and the molecular weights of JMI-229 tPA and Actilyse determined by their relative mobilities agianst known standards.

Under reducing conditions, samples of JMI-229 tPA migrate on SDS-PAGE as a single band of Mr 64,000 Da whilst Actilyse runs as an Mr 60,000-63,000 Da doublet. Plasmin treatment generated the 2-chain forms of each agent which, under reducing conditions in SDS-PAGE, resulted in two bands of Mr 31,000 Da and 39,000 Da for JMI-229 tPA, and two bands of Mr 33,000 Da and 37,000 Da for Actilyse.

Under non-reducing conditions, both the 1-chain and 2-

chain forms of JMI-229 tPA migrate as a single band with Mr 64,000 Da. Under the same conditions, both the 1-chain and 2-chain forms of Actilyse migrate as a doublet of Mr 60,000-63,000 Da.

The data reported here for Actilyse is in close agreement with published information (Pannekoek, H. et al, Fibrinolysis 2: 123-132, 1988).

(iv) Immunological Properties

In order properly to quantify JMI-229 tPA it was necessary to confirm whether a commercially-available ELISA for human tPA (Imulyse, Biopool) was capable of providing accurate and sensitive quantification. The results (Figure 2) indicate clearly that this is not the case. Thus, despite great similarity in the overall structure of rat and human tPAs, rat tPA apparently exhibits few shared epitopes with human tPA.

It was therefore necessary to develop an ELISA system specific for JMI-229 tPA. To this end, antibodies to JMI-229 tPA were raised in New Zealand white rabbits and IgG purified from the resultent antiserum by chromatography on Protein A-Sepharose (Pharmacia) following the manufacturer's instructions.

ELISA plates are coated with this IgG (5 ug/ml in 50m molar carbonate buffer, pH 9.6) overnight, after which the plates are washed and blocked with a 1% (w/v) solution of gelatin in PBS/0.01% (v/v) Tween 80. The plates are subsequently washed, and 100 ul samples of unknowns or standards added to each well. Following incubation for 2 hours, the plates are again washed and to each well is

added 100 ul of a 0.5 ug/ml solution of IgG conjugated with biotin and the plates further incubated for a period of 1 hour. Following extensive washing, 100 ul aliquots of streptavidin/biotin/horseradish peroxidase (Amersham) are added to each well, and the plate incubated for 30 minutes. Following washing of the plates, a solution of 50 mM sodium acetate containing 0.01% (w/v) 3,3',5,5' - tetramethylbenzidine and 0.015% (v/v) hydrogen peroxide is prepared and 100 ul added to each well. Colour development is allowed to proceed in the dark for about 20 minutes, after which the reaction is terminated by addition of 50 ul 4M H_2SO_4 to each well. Following mixing, the absorbance of each well is measured at 450 nm.

The comparative responses of JMI-229-tPA and Actilyse are shown in Figure 3. The immunological differences between JMI-229 tPA and Actilyse are again evident.

(ii) Isoelectric Point

The isoelectric points of JMI-229 tPA and Actilyse have been determined in 1.5 mm thickness polyacrylamide gels containing Ampholines, urea and NP40. Gels were prefocussed at 20 mA for 45 minutes and the temperature maintained at 10°C. Samples of JMI-229 tPA and Actilyse (each 15 ug) containing 1% (v/v) NP40 and 2.25 M urea were loaded on applicators 20 mm from the anode. The samples were focussed first at 20 mA for 20 minutes at a maximum voltage of 200 V, and then the voltage increased to 1,000 V for 3 hours. Gels were then fixed for 10 minutes in a solution comprising methanol (3.5% [v/v]), trichloroacetic acid (13% [w/v]), and acetic acid (10% [v/v]).

Both agents exhibit heterogeneity in this system. Under a variety of differing pH ranges (pH 3 - 10, pH 6.5. - 11, pH9 - 11 and pH 8 - 10.5) JMI-229 tPA migrated migrated as a series of six major bands in the range pH 8.9 -9.5. In contrast Actyilyse migrated a seven major bands in the range pH 6.6 - 8.5. The tPA from JMI-229 cells is therefore considerably more basic than Actilyse.

(iii) cDNA and Amino Acid Sequences

The amino acid sequence defining the structure of the tPA from JMI-229 cells has been elucidated. Cells expressing tPA were used as a source of mRNA from which a cDNA copy was prepared, using commercial kits and manufacturer's instructions (Amersham, UK). cDNA was size-fractionated by exclusion chromatography and cloned into a lambda GT phage, as described in "Molecular Cloning: A Laboratory Manual", Eds. T Maniatis, E.F. Fritsch & J Sambrook, published by Cold Spring Harbour Laboratory, 1982. Plaques were probed with two oligonucleotide probes a) 5' GGT AAG TTG TCT GAG TCT GTT CAT CTC TGC AGG 3', and b) 5'G CCA AGG GTG TGA GGT GAT GTC TGT GAA GAG 3', chosen as complementary to the gene for mouse tPA. Hybridising clones were subjected to three rounds of enrichment and four clones were subjected to cDNA extraction and sequencing. The sequence of clone PPA15 is shown in Table 1, together with the corresponding amino acid sequence. The three additional clones exhibited identical sequences.

Table 1 shows the complete cDNA sequence of tPA clone PPA15, together with the corresponding amino acid sequence. The sequence covers 2512 nucleotides and comprises a 5' untranslated region of 90 nucleotides, an

open reading frame of 1677 nucleotides, a 3' untranslated region of 737 nucleotides and a poly (A) tail. Column (a) indicates the nucleotide residue number; column (b) indicates the amino acid residue from the start of the open reading frame and column (c) indicates the amino acid residue from the N-terminal amino acid, marked *. Note that the true N-terminal amino acid, is Serine, as determined by direct protein sequencing, and not the Glycine at position -3 suggested by Ny, T., Leonardsson, G. & Hsueh, A.J.W., DNA, 7(10): 671-677, 1988.

The nucleotide sequence of JMI-229 tPA differs from that recently published for rat tPA (Ny et al, 1988) in 2 residues. One of these changes results in amino acid 348 being glutamic acid in JMI-229 tPA rather than lysine (a basic amino acid) in the sequence of Ny et al. This change is potentially highly significant, the net electrical charge being opposite for each of the reported residues.

The other nucleotide difference occurs in the non-coding region of the cDNA sequence, where cytosine replaces adenosine at residue 1921 of PPA15. This change may result in alterations to mRNA stability or translatability.

A significant proportion of the amino acid sequence has been confirmed by direct protein sequencing of the native protein, and of peptides derived by proteolytic cleavage, using an Applied Biosystems Model 477A gas-phase protein sequencer, following HPLC isolation of the protein fragments (Table 2). In Table 2, residues underlined with asterisks have been confirmed by protein sequencing.

Although the amino acid sequence of JMI-229 tPA is almost identical to that described for rat tPA (Ny et al, 1988) there has been no previous characterisation of the properties of the tPA derived from a non-cancerous rat cell line. The data described here clearly distinguishes this tPA from that previously described by Strickland et al (1983). Waller (1984) and Pollard (US 4,661,453). This difference may stem from the acid/base change at position 348, facilitating greater ionic interaction with fibrin, resulting in the enhanced binding properties detailed in the examples below. Additional differences in properties of the two types of tPA may be due to different states of glycosylation: the degree and nature of glycosylation are determined by the producing cell type, and these in turn determine the observed moleular weight and influence immunological properties.

The invention will be further described, by way of illustration, in the following description and Examples which refer to the accompanying drawings in which:

Figure 1 illustrates the result of fingerprint analysis of DNA from JMI-229, RLC and MRC-5;

Figures 2 and 3 are graphs illustrating the results of comparison JMI-229 tPA and Acitlyse in ELISA assays, with Figure 2 giving results for a commercial ELISA and Figure 3 results for an in-house ELISA.

Figure 4 is a graph illustrating binding to intact fibrin;

Figure 5 is a graph illustrating binding to degraded fibrin;

Figure 6 is a graph illustrating binding of 1-C JMI-229 tPA to fibrin;

Figure 7 is a pair of graphs illustrating the time course of tPA binding to preformed clots;

Figure 8 is a graph illustrating the % of applied antigen bound over 1 hour;

Figure 9 is a graph illustrating tPA induced lysis for cross-linked plasma clot;

Figure 10 is a graph illustating the clearance of active tPA from rabbits, showing circulating activities of JMI-229 tPA and actilyse following 30k IU/kg bolus dose, with data presented as mean +/- sem;

Figure 11 is a pair of graphs identifying components of the clearance curves presented in Figure 10, showing curve peeling to separate alpha and beta phases; and

Figure 12 is a graph illustrating the efficacy of tPA in a rabbit model, showing activities of JMI-229 tPA and actilyse in a rabbit arterio-venous shunt model, with dose applied as 10% bolus followed by a 3 hour infusion.

Production of tPA from JMI-229 Cells

Culture of JMI-229 Cells

The proceedure used to culture JMI-229 cells was similar in principle to that described in International Application No PCT/GB88/00758 (Publication No.

WO89/02917). Cells were grown in suspension in Excell 300 medium (J R Scientific, Woodland, California, USA) (serum-free) first at 1L and then 10L scale. This latter culture was used to inoculate a 100L bioreactor vessel containing 5 g/L Dormacell 2.6 microcarriers. Following 72 hours of cell growth at 36.5°C, the medium was replaced with Excell 300 containing 10 ug/ml concanavalin A which enhances tPA production from these cells. After 48 hours the enzyme-containing medium was harvested, and fresh medium containing 5 ug/ml concanavalin A added. This process was repeated twice more.

The culture process can alternatively be carried out in roller bottles.

Purification of JMI-229 tPA

The tPA was purified by a modification of the method of Rijken and Collen (Journal of Biological Chemistry, (1981) 7035-7041). All procedures are carried out aseptically in order to generate a pyrogen-free product.

Enzyme-containing medium (50-400L), from either roller or bioreactor culture is adjusted to 1% (v/v) Tween 80 and 0.2% (v/v) chloroform. It is then passed through a 0.2 um polypropylene filter and loaded onto a column containing 3,000 ml Chelating Sepharose FF, previously loaded with Zn^{2+}. Column operation is controlled by means of a process controller which automatically switches from load to wash to elution. The wash buffer comprises 50mM Tris HCl pH 7.5, 1M NaCl, 0.01% (v/v) Tween 80, 0.02% chloroform (buffer I). Elution of bound protein is with buffer I containing 50mM Imidazole.

Eluted protein is applied to a column containing 300 ml of concanavalin A-Sepharose, which is then washed with buffer I. Bound protein is eluted by means of a linear gradient from buffer I to 50mM Tris HCl pH 7.5, 0.5 M potassium thiocyanate, 1 M alpha-methylmannoside, 0.01% (v/v) Tween 80, 0.2% chloroform (buffer II). Following the gradient, the column is further washed with buffer II to ensure complete protein removal.

Eluted tPA is then diluted 10-fold and applied to a column containing 60 ml Lysine Sepharose 4B. The column is then washed with 20 mM potassium phosphate pH 7.5, 0.01% Tween 80, 0.2% chloroform (buffer III), and finally the pure tPA eluted by a linear gradient from buffer II to buffer III additionally containing 2M potassium thiocyanate (buffer IV).

The eluted tPA is then concentrated by pressure filtration to a level of 1 mg/ml and stored at -70°C. Prior to use it is thawed and desalted by gel filtration in the presence of 200 mM potassium phosphate pH 7.5, 0.1% (v/v) Tween 80.

Examples

Binding of tPA to Fibrin Clots

A: Forming Clot Model

The experimental approach used here is analogous to that used by Higgins & Vehar (Biochemistry (1987) 26 7786-7791) who have characterised the binding to fibrin of both the one-chain and two-chain versions of rtPA. The affinity of both forms is enhanced if the fibrin is first partially

degraded with plasmin prior to clot formation. This enhanced binding, due to the generation of new high affinity binding sites for tPA, may have a physiological role.

These experiments have been repeated using JMI-229 tPA with Actilyse used as a control to represent the current pharmacological agent.

Example 1. Binding to Intact Fibrin

In this example, fixed concentrations (20 IU/ml) of tPA have been mixed with a range of human fibrinogen concentrations (0.05-1mg/ml in 50 mM Tris HCl pH 7.5, 100 mM NaCl, 0.01% (v/v) Tween 80, 0.1% (w/v) human serum albumin (buffer V)) and the solutions clotted with human thrombin (1 NIH unit/ml). Following incubation at 37°C for 15 minutes, the clots were compacted by centrifugation at 11,000 x g for 5 minutes and the supernatants removed. tPA remaining in these supernatants was quantitated by an ELISA specific for each agent (Biopool kit for Actilyse, in-house method for JMI-229 tPA). Results were confirmed by estimation of tPA activity in a clot lysis assay or by S2251 cleavage.

JMI-229 tPA was prepared in the one-chain form by inclusion of aprotinin during isolation, and the two-chain material was prepared by subsequent incubation of the one-chain form with plasmin-Sepharose.

Preliminary experiments (not shown) indicated that maximal binding occured within 15 minutes of clot formation with no significant increase up to 60 minutes. 15 minute incubations were then used throughout the work.

Results of binding to intact fibrin are presented in Table 3 and Figure 4 from which it is immediately clear that the degree of binding exhibited by JMI-229 tPA, in both its one- and two-chain forms, is considerably greater than that exhibited by Actilyse (which is approximately 70% in the one-chain form).

(The reduced protein level evident for two-chain JMI-229 tPA is because, unlike human tPA, the two-chain version is considerably more active in a clot lysis assay.)

Example 2. Binding to Plasmin-Degraded Fibrin

This is an analogous to Example 1 with 20 IU/ml of each agent, except that in this case fibrinogen was first part-degraded by incubation with plasmin. The results are shown in Figure 5 and Table 4. Exceptionally high binding of the two-chain JMI-229 tPA is evident when compared with Actilyse.

Example 3. Binding of One-Chain JMI-229 tPA to Intact & Degraded Fibrin

For this direct comparison of the binding of one-chain JMI-229 tPA to either intact or plasmin-degraded fibrin, only 10 IU/ml enzyme was used. All other conditions were as described for Example 1.

The exceptional affinity of JMI-229 tPA for degraded fibrin was such that the amount remaining in the supernatant was below the sensitivity of the ELISA (less than 1 ng/ml) at all fibrin concentrations, when degraded fibrin was used (Table 5 and Figure 6).

B: Preformed Clot Model

In order to verify whether the enhanced binding properties of JMI-229 tPA found in the above model would be likely to apply in a pharmacological situation, another model was set up involving a pre-formed clot. This was again prepared using plasminogen-free human fibrin at 3 mg/ml in buffer V. Uniform clots were formed in tubing (4 mm internal diameter) and were initiated by addition of 8 IU/ml human thrombin. Following clot formation, 1cm lengths of tubing were cut, and the clots expressed from the tubing. Prior to use they were incubated for 17h at 4°C in buffer V.

Example 4

Clots were incubated at 37°C in 0.5ml buffer V containing 100ng either JMI-229 tPA or Actilyse. At intervals the clots were removed and the amount of agent remaining free in solution estimated by ELISA. As shown in Figure 7, there is a steady increase in the amount of agent bound over a 6 hour period. Incubation for longer periods does not lead to further increases in amounts bound.

The difference between the two agents is clearly evident in Figure 7, showing a significantly increased proportion of JMI-229 tPA bound to the clots compared to Actilyse, at all time points.

The observed increase in binding over 6 hours probably reflects a combination of two factors - agent binding to the surface of the clot, and agent diffusing into the clot. To be pharmacologically valid, the important factor

is binding to the clot surface. This was estimated by comparing the proportions of each agent bound over a fixed period of one hour, as detailed in Example 5, below.

Example 5

To assess the amount of agent bound to the clot surface, replicate clots were incubated in 0.5ml volumes of buffer V containing a wide range of agent concentrations. Following incubation at 37°C for one hour, clots were removed and the proportion of agent left remaining in the solution determined by ELISA.

The results (Figure 8) show a consistently higher proportion of JMI-229 tPA bound to the clot than Actilyse, over the very wide range of agent concentrations examined.

In Vitro Clot Lysis

The ability to JMI-229 tPA to lyse human plasma clots has been examined.

In the example described below, a significantly enhanced ability to lyse fully cross-linked clots is demonstrated by JMI-229 tPA compared with human tPA.

Example 6

Freshly drawn human blood was citrated and platelet-poor plasma prepared by centrifugation at 3,500g for 15 minutes. The separated plasma was pooled and a trace of ^{125}I-human fibrinogen added. Aliquots (0.5ml) of plasma were diluted to 4.9ml with HEPES/NaCl/Tween buffer pH 7.4

and clotted with 100 ul bovine thrombin (125 Iu/Ml, 0.25 M $CaCl_2$) in the presence of glass wool. Clots were then incubated at 37°C for 17 hours.

A proportion of the clots were washed and used to assess the amount of bound label. The remainder were incubated with variable quantities of tPA and at regular intervals clots were separated from the supernatant, and the proportion of remaining fibrin determined. Each measurement at each time interval was performed in triplicate, and the time at which 50% clot lysis was achieved determined from plots of % lysis vs. time for each tPA concentration.

The results (Figure 9) show a significantly enhanced rate of lysis with JMI-229 tPA. This can also be envisaged as indicating that a significantly reduced dose of JMI-229 tPA would be required to induce the same degree of clot lysis in the same time, as compared with human tPA.

In Vivo Half Life

Tissue-type plasminogen activator has a very short half-life in the circulation and is thought to be eliminated almost exclusively via the liver. (For review, see Krause, J. (1988) Fibrinolysis 2: 133-142.) It follows, therefore, that any agent which exhibits an increased circulating half-life would permit the administration of reduced doses.

Example 7. Circulating Half-Life of JMI-229 tPA in rabbits.

In order to determine if JMI-229 tPA and Actilyse

exhibited very similar clearance half-lives, two groups each comprising six New Zealand white rabbits were used. Each rabbit received a bolus dose of 30,000 IU/Kg delivered into the jugular vein. Blood samples were removed from a femoral vein every minute and acidified to prevent further inactivation of the tPAs. Resultant plasma samples were assayed enzymically in order to determine the levels of active circulating enzyme.

The data are presented in Figures 10 and 11 from which the significantly increased half-life of JMI-229 tPA is apparent. The decay of both agents is apparently biphasic, comprising an initial rapid alpha phase followed by a slower-decaying beta-phase. However, the characteristics of these decay curves differ significantly between the agents.

For JMI-229 tPA, the rapid alpha decay ($t_{\frac{1}{2}}$=0.58 minutes) is no longer significant after about 3 minutes. There is a very marked beta-phase decay exhibiting a significantly longer $t_{\frac{1}{2}}$ (3.57 minutes) which accounts for the bulk of the decay curve. In contrast, Actilyse has a slower-decaying alpha phase ($t_{\frac{1}{2}}$=1.03 minutes) which accounts for the bulk of the decay, followed by only a minor beta-phase decay with $t_{\frac{1}{2}}$ of 2.31 minutes. These data are summarised in Table 6.

Efficacy in vivo

In order to demonstrate the efficacy of JMI-229 tPA in vivo, it has been compaated with Actilyse in a rabbit arterio-venous shunt model. In this model, a pre-counted radiolabelled blood clot is held in the rabbit circulation for a period of four hours, during which the tPA is

administered. At the end of this period the clot is removed, the remaining radioactivity determined and the difference between this value and the original amount of radioactivity in the clot indicates the degree of clot lysis which has occured.

Example 8. Efficacy of JMI-229 tPA in a Rabbit Model

New Zealand white rabbits in the weight range 2-3 kg were used. Each was anaesthetised with an intramuscular injection of 3 mg/kg Hypnorm and an intraperitoneal injection of 2.5 mg/kg Valium.

Both femoral veins were catheterised with 'pink' gauge Portex cannulae to allow infusion of test or control compounds and to enable blood samples to be taken. Thyroidal uptake of ^{125}I was blocked by injection of 0.5 ml 2% (w/v) sodium iodide solution into the femoral vein. An external jugular vein was exposed through a paramedial incision in the neck. The vein was then cannulated using a 'red' gauge Portex cannula and washed through with 2 mls of heparinised saline (20 iu/ml) to maintain cannula efficiency. The carotid artery was also cleared and cannulated with a 'red' guage Portex cannula and washed through as for the vein.

The thrombus was produced in a shortened 1 ml syringe barrel (cut off at the 0.4 ml mark). 10ul of ^{125}I labelled fibrinogen was accurately pipetted in to a gamma counting tube, and 1 ml of fresh rabbit blood, withdrawn from the femoral vein, added to the tube and quickly mixed. 250 ul of this mixture was then aspirated into a 1 ml syringe containing 100 ul of 25mM $CaCl_2$ and 1 NIHU thrombin, inverted and transferred to the shortened

syringe barrel containing a woollen thread. The shortened barrel (with a 3-way tap preventing loss of material) was then incubated at 37°C for 30 minutes.

The syringe barrel was then washed with 0.9% (w/v) sodium chloride (2 lots of 2 ml) to remove any non-clotted radioactivity. The barrel and clot were then placed in a clean gamma counter tube and counted for 5 minutes using a Packard Cobra gamma counter. The syringe barrel was then linked via the luer fitting to the venous side cannula and via silicone tubing to the arterial cannula. The clot was held in place by a woollen thread.

5 minutes before the shunt was put in place 400 iu/kg heparin was administered via the femoral vein. The clot was then allowed to age for 30 minutes, blood samples removed and the test or control compound given. This was at 2 ml/kg/hr as a 10% bolus followed by a 3 hour infusion into the femoral vein. 4 hours after the start of infusion the syringe barrel was removed and the remaining radioactivity counted to assess the extent of the residual clot.

The efficacy of JMI-229 tPA was found to be considerably greater than that of Actilyse at low doses (Figure 12) as predicted from the fibrin-binding and <u>in vitro</u> clot lysis studies described in the preceding examples. It would be expected for these rabbit efficacy results to be repeated in a human clinical situation, where the reduced doses required for JMI-229 tPA should lead to minimal perturbation of the haemostatic balance and the diminution of side effects associated with treatment with Actilyse or other thrombolytic agents.

TABLE 1.

COMPLETE cDNA SEQUENCE, AND RESULTING AMINO ACID SEQUENCE, FOR tPA DERIVED FROM JMI-229 CELLS

(a)		(b)	(c)
1	GAGCTAATCA GCTCAGCGCC AAGGAGAAGC CCCTGCGAGA GTGGGAAAGA AGCAAGCAAG		
	Met Lys Gly Glu Leu Leu Cys Val Leu Leu	10	
61	GCACGGGACA CGGAAGAAAC GGGGAGCAAA ATG AAG GGA GAG CTG TTG TGC GTC CTG CTG		
	Leu Cys Gly Val Ala Phe Thr Leu Pro Asp Gln Gly Ile His Arg Arg Phe Arg Arg Gly	30	
121	CTT TGT GGA GTG GCG TTC ACG TTG CCT GAC CAG GGA ATA CAC AGG AGG TTC AGA AGA GGA		
* N - TERMINUS	*		
	Ala Arg Ser Tyr Arg Ala Thr Cys Arg Asp Glu Gln Thr Gln Thr Thr Tyr Gln Gln His	50	18
181	GCT CGG TCC TAC AGA GCG ACC TGC AGA GAT GAA CAG ACT CAG ACA ACT TAC CAA CAG CAT		
	Gln Ser Trp Leu Arg Pro Met Leu Arg Gly Asn Arg Val Glu Tyr Cys Arg Cys Asn Ser	70	38
241	CAG TCA TGG CTA CGT CCC ATG CTC AGA GGC AAT CGG GTG GAA TAC TGC CGG TGC AAC AGC		
	Gly Leu Ala Gln Cys His Ser Val Pro Val Arg Ser Cys Ser Glu Pro Arg Cys Phe Asn	90	58
301	GGC CTG GCA CAG TGC CAC TCG GTG CCC GTC CGA AGT TGC AGT GAA CCG AGA TGC TTC AAT		
	Gly Gly Thr Cys Gln Gln Ala Leu Tyr Phe Ser Asp Phe Val Cys Gln Cys Pro Asp Gly	110	78
361	GGG GGT ACG TGT CAG CAG GCC CTG TAT TTC TCT GAC TTC GTC TGC CAG TGC CCT GAC GGA		
	Phe Val Gly Lys Arg Cys Asp Ile Asp Thr Arg Ala Thr Cys Phe Glu Gly Gln Gly Ile	130	98
421	TTT GTT GGG AAA CGC TGT GAT ATA GAT ACC AGA GCA ACC TGC TTC GAG GGC CAG GGC ATC		
	Thr Tyr Arg Gly Thr Trp Ser Thr Ala Glu Asn Gly Ala Glu Cys Ile Asn Trp Asn Ser	150	118
481	ACC TAC AGA GGC ACA TGG AGC ACA GCA GAA AAT GGG GCT GAA TGC ATC AAC TGG AAT AGC		
	Ser Ala Leu Ser Gln Lys Pro Tyr Ser Ala Arg Arg Pro Asn Ala Ile Lys Leu Gly Leu	170	138
541	AGT GCC CTG TCC CAG AAG CCC TAC AGT GCA AGG AGG CCA AAT GCC ATC AAG CTG GGC CTT		
	Gly Asn His Asn Tyr Cys Arg Asn Pro Asp Arg Asp Val Lys Pro Trp Cys Tyr Val Phe	190	158
601	GGG AAT CAC AAT TAC TGC AGA AAC CCA GAC CGA GAC GTG AAG CCC TGG TGC TAT GTC TTT		

TABLE 1 continued

(a)		(b)	(c)
	Lys Ala Gly Lys Tyr Thr Thr Glu Phe Cys Ser Thr Pro Ala Cys Pro Lys Gly Pro Thr	210	178
661	AAG GCA GGG AAG TAT ACC ACG GAG TTC TGC AGC ACG CCG GCT TGC CCT AAG GGA CCA ACT		
	Glu Asp Cys Tyr Val Gly Lys Gly Val Thr Tyr Arg Gly Thr His Ser Phe Thr Thr Ser	230	198
721	GAG GAC TGC TAT GTT GGA AAA GGT GTG ACT TAC CGT GGC ACC CAC AGC TTT ACC ACA TCC		
	Lys Ala Ser Cys Leu Pro Trp Asn Ser Met Ile Leu Ile Gly Lys Thr Tyr Thr Ala Trp	250	218
781	AAG GCC TCC TGC CTC CCA TGG AAT TCC ATG ATC CTG ATA GGC AAG ACT TAC ACA GCG TGG		
	Arg Ala Asn Ser Gln Ala Leu Gly Leu Gly Arg His Asn Tyr Cys Arg Asn Pro Asp Gly	270	238
841	AGG GCC AAC TCC CAG GCA CTT GGC CTG GGC AGA CAC AAT TAT TGC CGG AAC CCA GAT GGG		
	Asp Ala Lys Pro Trp Cys His Val Met Lys Asp Arg Lys Leu Thr Trp Glu Tyr Cys Asp	290	258
901	GAT GCC AAA CCT TGG TGC CAC GTG ATG AAG GAC CGA AAG CTG ACA TGG GAA TAT TGC GAC		
	Met Ser Pro Cys Ser Thr Cys Gly Leu Arg Gln Tyr Lys Gln Pro Gln Phe Arg Ile Lys	310	278
961	ATG TCC CCA TGC TCC ACC TGC GGC CTG AGG CAA TAC AAA CAG CCT CAG TTT CGA ATT AAA		
	Gly Gly Leu Phe Thr Asp Ile Thr Ser His Pro Trp Gln Ala Ala Ile Phe Val Lys Asn	330	298
1021	GGA GGA CTC TTC ACA GAC ATC ACC TCA CAC CCT TGG CAG GCC GCC ATC TTT GTC AAG AAC		
	Lys Arg Ser Pro Gly Glu Arg Phe Leu Cys Gly Gly Val Leu Ile Ser Ser Cys Trp Val	350	318
1081	AAG AGG TCT CCA GGA GAG AGA TTC CTG TGT GGA GGG GTG CTG ATC AGT TCC TGC TGG GTG		
	Leu Ser Ala Ala His Cys Phe Val Glu Arg Phe Pro Pro His His Leu Lys Val Val Leu	370	338
1141	CTA TCT GCC GCC CAC TGC TTT GTA GAG AGG TTT CCA CCC CAT CAT CTT AAA GTG GTC TTG		
	Gly Arg Thr Tyr Arg Val Val Pro Gly Glu Glu Glu Gln Thr Phe Glu Ile Glu Lys Tyr	390	358
1201	GGC AGA ACA TAC AGA GTG GTC CCT GGA GAG GAG GAG CAG ACA TTC GAG ATC GAA AAG TAC		
	Ile Val His Lys Glu Phe Asp Asp Asp Thr Tyr Asp Asn Asp Ile Ala Leu Leu Gln Leu	410	378
1261	ATA GTC CAT AAG GAA TTT GAT GAC GAC ACT TAT GAC AAT GAC ATC GCA TTA CTG CAG CTG		
	Arg Ser Asp Ser Ser Gln Cys Ala Gln Glu Ser Ser Ser Val Gly Thr Ala Cys Leu Pro	430	398
1321	AGG TCA GAT TCC AGT CAG TGT GCC CAG GAG AGC AGT TCT GTC GGC ACT GCC TGC CTC CCT		

TABLE 1 continued

(a)		(b)	(c)
	Asp Pro Asp Val Gln Leu Pro Asp Trp Thr Glu Cys Glu Leu Ser Gly Tyr Gly Lys His	450	418
1381	GAC CCC GAC GTA CAG CTC CCT GAC TGG ACA GAG TGT GAG CTT TCT GGC TAC GGC AAG CAT		
	Glu Ala Ser Ser Pro Phe Phe Ser Asp Arg Leu Lys Glu Ala His Val Arg Leu Tyr Pro	470	438
1441	GAG GCA TCC TCT CCT TTC TTC TCT GAC CGG CTG AAG GAG GCT CAC GTC AGA CTG TAT CCG		
	Ser Ser Arg Cys Thr Ser Gln His Leu Phe Asn Lys Thr Ile Thr Ser Asn Met Leu Cys	490	458
1501	TCC AGC CGC TGT ACC TCA CAG CAT CTG TTT AAC AAA ACC ATC ACG AGC AAC ATG CTG TGT		
	Ala Gly Asp Thr Arg Thr Gly Gly Asn Gln Asp Val His Asp Ala Cys Gln Gly Asp Ser	510	478
1561	GCA GGA GAC ACC CGA ACT GGG GGC AAC CAA GAC GTC CAT GAC GCG TGC CAG GGT GAC TCA		
	Gly Gly Pro Leu Val Cys Met Ile Asp Lys Arg Met Thr Leu Leu Gly Ile Ile Ser Trp	530	498
1621	GGA GGC CCT CTG GTG TGC ATG ATC GAT AAG CGG ATG ACT TTA CTG GGC ATC ATC AGC TGG		
	Gly Leu Gly Cys Gly Gln Lys Asp Val Pro Gly Ile Tyr Thr Lys Val Thr Asn Tyr Leu	550	518
1681	GGC CTC GGC TGT GGG CAG AAG GAC GTG CCA GGG ATA TAC ACA AAG GTC ACT AAT TAC CTG		
	Asn Trp Ile Gln Asp Asn Met Lys Gln	559	527
1741	AAC TGG ATC CAA GAC AAC ATG AAG CAA TGA CAAAGAAAGC CCAGCTCCTT AAACCCAGAG		
1801	GACCTGCCTT CCTCTTCCTC TTCCACAGAA GATACGCCTG AAAGGCCAAG CGTTCTTCGC		
1861	AGGCTCGTCC TCCTGAGCTG CCGCTCAGCA GAGGGAGTGA CAGTCTTTAG GCACAGACAG		
1921	CGTTTACTTT GTGACAGGTA CTTCACAAAC TTGTACGTTT TAAGGGTGAA GGTCTGACTT		
1981	TAGAATCAGT TCTGTCAGAT GAGATGACAG GGAAATGCCA ACCTTCCTAT AACTCTAAGA		
2041	TTTTAAAAAG AGAAGTAGAC CAAAGTCCAC CCTTCCTGGA CCACTATTTT GTACACTGAA		
2101	CCACAAGATC GTGTCTCAAC AGTGAAATAC AACTTGATCT TTCAGGAGTA AAAGTCTGCA		
2161	CTGAGGACAA GAATGTGTTT TTATAGTTAC ACAGGGGCCC AGCATGGCCT CCAAGAGAAG		
2221	GAAGGGGTTA GCTGATCAGA CCACAGCCCC CTAAAACCCT TGAAATCAAA TATTCCCCAT		

TABLE 1 continued

(a)

2281 CCTCCCACAA TCCTCAACTC TTGGGGCATA TCCCTTTGTA CACAGTGTAG ATGTCTTTTT

2341 CTTTATAAAC TTTCCAGATG GCTGGGAGAA CTGTATGATT TTAATATTCG ATGAAATGAC

2401 ACTAGTATAT TTATATTTGA ATCTATTTAG TTTTTACTGT GTTACTAGAA CTCTGTATTA

2461 TGCTGTACTG AAATAATAAA TTCCAAGGTA TTTTTCACAC TTTTAAAAAA AA

TABLE 2

RAT tPA AMINO ACID SEQUENCE - REGIONS CONFIRMED BY PROTEIN SEQUENCING

	-3 -2 -1 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
	GLY ALA ARG SER TYR ARG ALA THR CYS ARG ASP GLU GLN THR GLN THR THR TYR GLN GLN
	**********
18	HIS GLN SER TRP LEU ARG PRO MET LEU ARG GLY ASN ARG VAL GLU TYR CYS ARG CYS ASN
	********** **********
38	SER GLY LEU ALA GLN CYS HIS SER VAL PRO VAL ARG SER CYS SER GLU PRO ARG CYS PHE
	**********
58	ASN GLY GLY THR CYS GLN GLN ALA LEU TYR PHE SER ASP PHE VAL CYS GLN CYS PRO ASP
78	GLY PHE VAL GLY LYS ARG CYS ASP ILE ASP THR ARG ALA THR CYS PHE GLU GLY GLN GLY
	**********
98	ILE THR TYR ARG GLY THR TRP SER THR ALA GLU ASN GLY ALA GLU CYS ILE ASN TRP ASN
	**********
118	SER SER ALA LEU SER GLN LYS PRO TYR SER ALA ARG ARG PRO ASN ALA ILE LYS LEU GLY
138	LEU GLY ASN HIS ASN TYR CYS ARG ASN PRO ASP ARG ASP VAL LYS PRO TRP CYS TYR VAL
158	PHE LYS ALA GLY LYS TYR THR THR GLU PHE CYS SER THR PRO ALA CYS PRO LYS GLY PRO
	**********
178	THR GLU ASP CYS TYR VAL GLY LYS GLY VAL THR TYR ARG GLY THR HIS SER PHE THR THR
198	SER LYS ALA SER CYS LEU PRO TRP ASN SER MET ILE LEU ILE GLY LYS THR TYR THR ALA
218	TRP ARG ALA ASN SER GLN ALA LEU GLY LEU GLY ARG HIS ASN TYR CYS ARG ASN PRO ASP
238	GLY ASP ALA LYS PRO TRP CYS HIS VAL MET LYS ASP ARG LYS LEU THR TRP GLU TYR CYS
	**********
258	ASP MET SER PRO CYS SER THR CYS GLY LEU ARG GLN TYR LYS GLN PRO GLN PHE ARG ILE
	********** ***
278	LYS GLY GLY LEU PHE THR ASP ILE THR SER HIS PRO TRP GLN ALA ALA ILE PHE VAL LYS
	**********
298	ASN LYS ARG SER PRO GLY GLU ARG PHE LEU CYS GLY GLY VAL LEU ILE SER SER CYS TRP
	**********
318	VAL LEU SER ALA ALA HIS CYS PHE VAL GLU ARG PHE PRO PRO HIS HIS LEU LYS VAL VAL
338	LEU GLY ARG THR TYR ARG VAL VAL PRO GLY GLU GLU GLU GLN THR PHE GLU ILE GLU LYS
358	TYR ILE VAL HIS LYS GLU PHE ASP ASP ASP THR TYR ASP ASN ASP ILE ALA LEU LEU GLN
378	LEU ARG SER ASP SER SER GLN CYS ALA GLN GLU SER SER SER VAL GLY THR ALA CYS LEU
	**********
398	PRO ASP PRO ASP VAL GLN LEU PRO ASP TRP THR GLU CYS GLU LEU SER GLY TYR GLY LYS
	********** **********
418	HIS GLU ALA SER SER PRO PHE PHE SER ASP ARG LEU LYS GLU ALA HIS VAL ARG LEU TYR
	********** **********
438	PRO SER SER ARG CYS THR SER GLN HIS LEU PHE ASN LYS THR ILE THR SER ASN MET LEU
	**********
458	CYS ALA GLY ASP THR ARG THR GLY GLY ASN GLN ASP VAL HIS ASP ALA CYS GLN GLY ASP
478	SER GLY GLY PRO LEU VAL CYS MET ILE ASP LYS ARG MET THR LEU LEU GLY ILE ILE SER
498	TRP GLY LEU GLY CYS GLY GLN LYS ASP VAL PRO GLY ILE TYR THR LYS VAL THR ASN TYR
518	LEU ASN TRP ILE GLN ASP ASN MET LYS GLN

TABLE 3

BINDING TO INTACT FIBRIN

(Fibrinogen) (mg/ml)	ACTILYSE ng/ml in s/natant	ACTILYSE % Bound	JMI-299 tPA 1-CHAIN ng/ml in s/natant	JMI-299 tPA 1-CHAIN % Bound	JMI-299 tPA 2-CHAIN ng/ml in s/natant	JMI-299 tPA 2-CHAIN % Bound
1.0	9.67	78.4	<1	>96	1.71	92.4
0.5	13.17	70.5	<1	>96	2.39	89.4
0.2	17.6	60.6	1.91	95.4	5.37	76.3
0.1	22.35	50.0	3.56	91.4	8.37	63.0
0.05	37.3	16.6	3.96	90.5	6.33	72.0
Control	44.7		41.63		22.63	

TABLE 4

BINDING TO DEGRADED FIBRIN

[Fibrinogen] (mg/ml)	ACTILYSE ng/ml in s/natant	% Bound	JMI-229 tPA 2-CHAIN ng/ml in s/natant	% Bound
1	7.4	84.3	2.17	91.1
0.5	8.51	81	2.86	88.2
0.2	14.55	67.4	4.21	82.7
0.1	22.44	50.8	4.37	82
0.05	25.42	43.1	4.03	83.4
Control	44.7		24.83	

TABLE 5

BINDING OF 1-CHAIN JMI-229 tPA TO INTACT & DEGRADED FIBRIN

[Fibrinogen] (mg/ml)	INTACT ng/ml in s/natant	% Bound	DEGRADED ng/ml in s/natant	% Bound
1.0	<1	>96	<1	>96
0.5	<1	>96	<1	>96
0.2	<1	>96	<1	>96
0.1	1.04	93.5	<1	>96
0.05	1.77	91.8	<1	>96
Control	21.53		25.22	

TABLE 6

Clearance Data for JMI-229 tPA and Actilyse in Rabbits

Agent	Initial Volume of Distribution (ml)	$t_{\frac{1}{2}}$ a (min)	$t_{\frac{1}{2}}$ b (min)	Area under curve	Clearance (ml/min/kg)
JMI-229-tPA	151.45	0.58	3.57	464	64.6
Actilyse	200.48	1.03	2.31	320	93.5

Both agents applied as a 30,000 IU/kg bolus dose.

Claims

1. A pharmaceutical preparation comprising tPA derived from any non-cancerous rat cell line, the tPA having serine as the N-terminal amino acid and glutamic acid at position 348 counted from the N-terminal end of the molecule.

2. A pharmaceutical preparation comprising tPA derived from the gene for rat tPA, cloned and expressed in any cell line, the tPA having serine as the N-terminal amino acid and glutamic acid at position 348 counted from the N-terminal end of the molecule.

3. A pharmaceutical preparation comprising tPA having an amino acid sequence substantially identical to that for rat tPA derived from rat cells of non-cancerous origin, the tPA having serine as the N-terminal amino acid and glutamic acid at position 348 counted from the N-terminal end of the molecule.

4. A pharmaceutical preparation comprising tPA derived from the cell line JMI-229.

5. A pharmaceutical preparation comprising tPA derived from the gene for tPA in the cell line JMI-229 cloned and expressed in any other cell line.

6. A pharmaceutical preparation according to any one of the preceding claims, wherein the tPA is in the one-chain form.

7. A pharmaceutical preparation according to any one of claims 1 to 5 wherein the tPA is in the two-chain form.

8. A method of treating conditions which derive from obstruction of blood vessels by fibrin clots, comprising administering an effective amount of a preparation in accordance with any one of the preceding claims.

9. A method of producing tPA, which comprises culturing cells of the cell line JMI-229 and isolating a tPA-containing fraction from the culture.

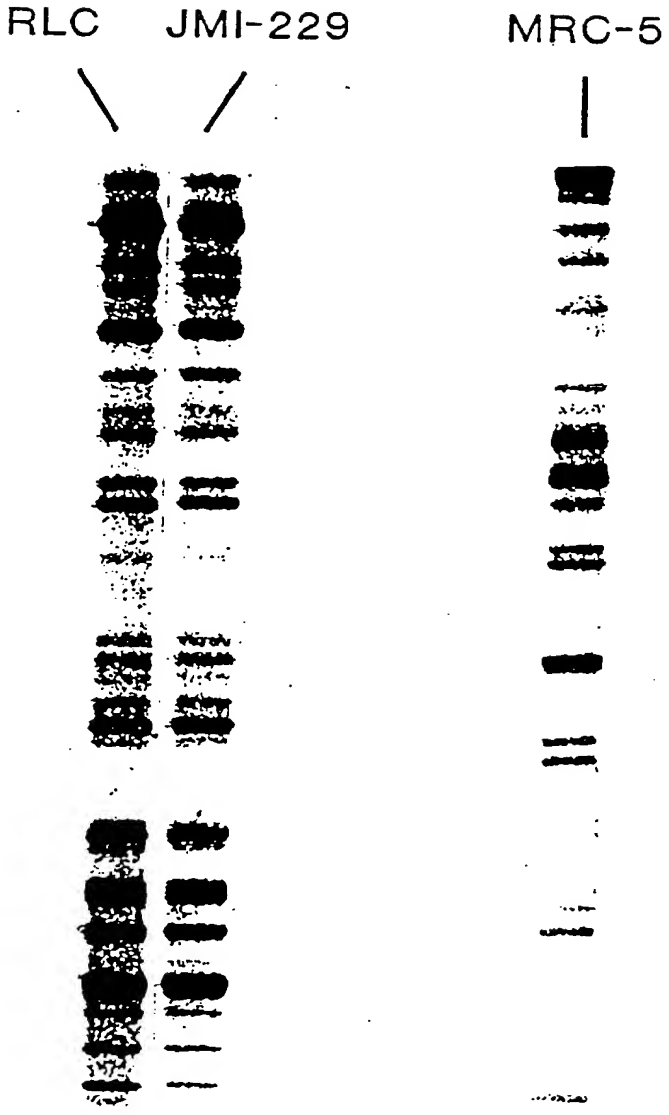

Fig. 1

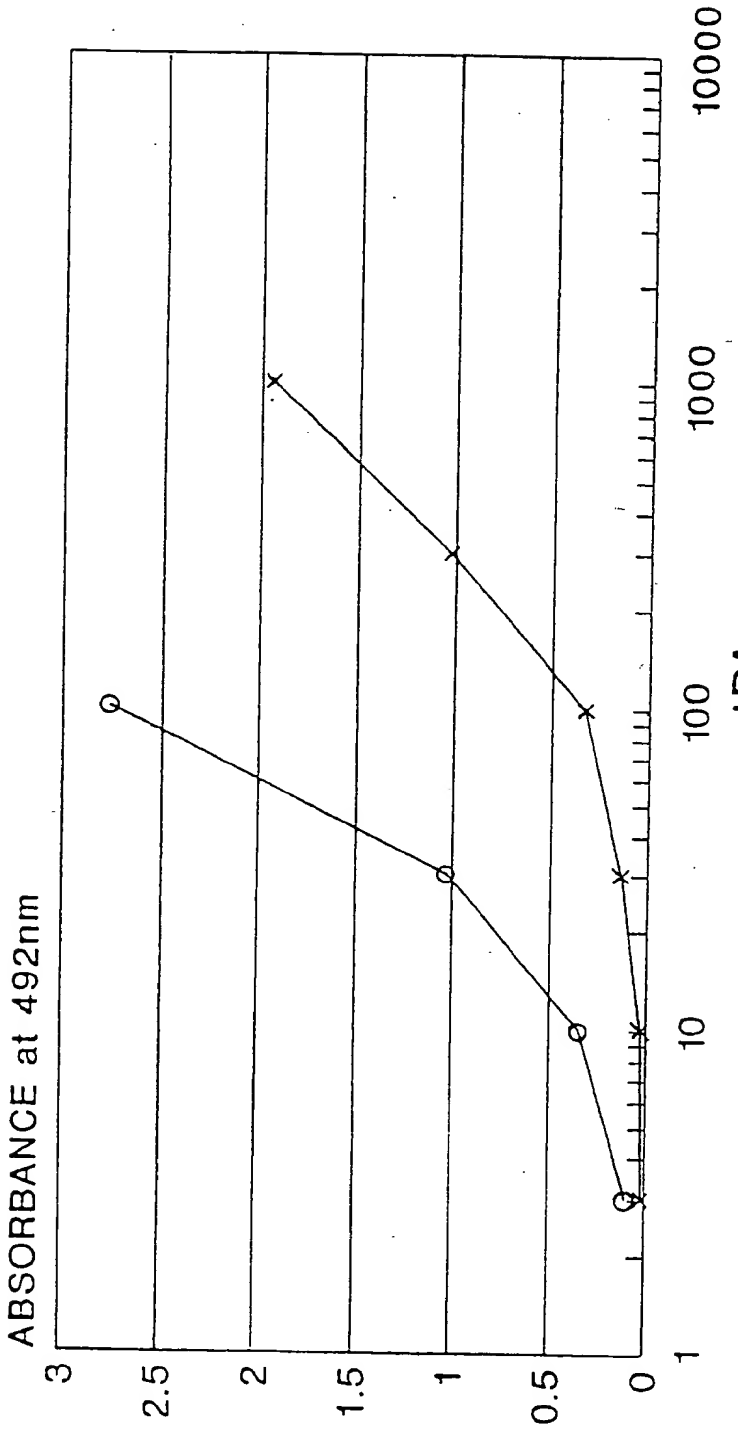
ABSORBANCE at 492nm
3
2.5
2
1.5
1
0.5
0
1
10
100
1000
10000
ng tPA
ACTILYSE
JMI-229 tPA

Fig. 2

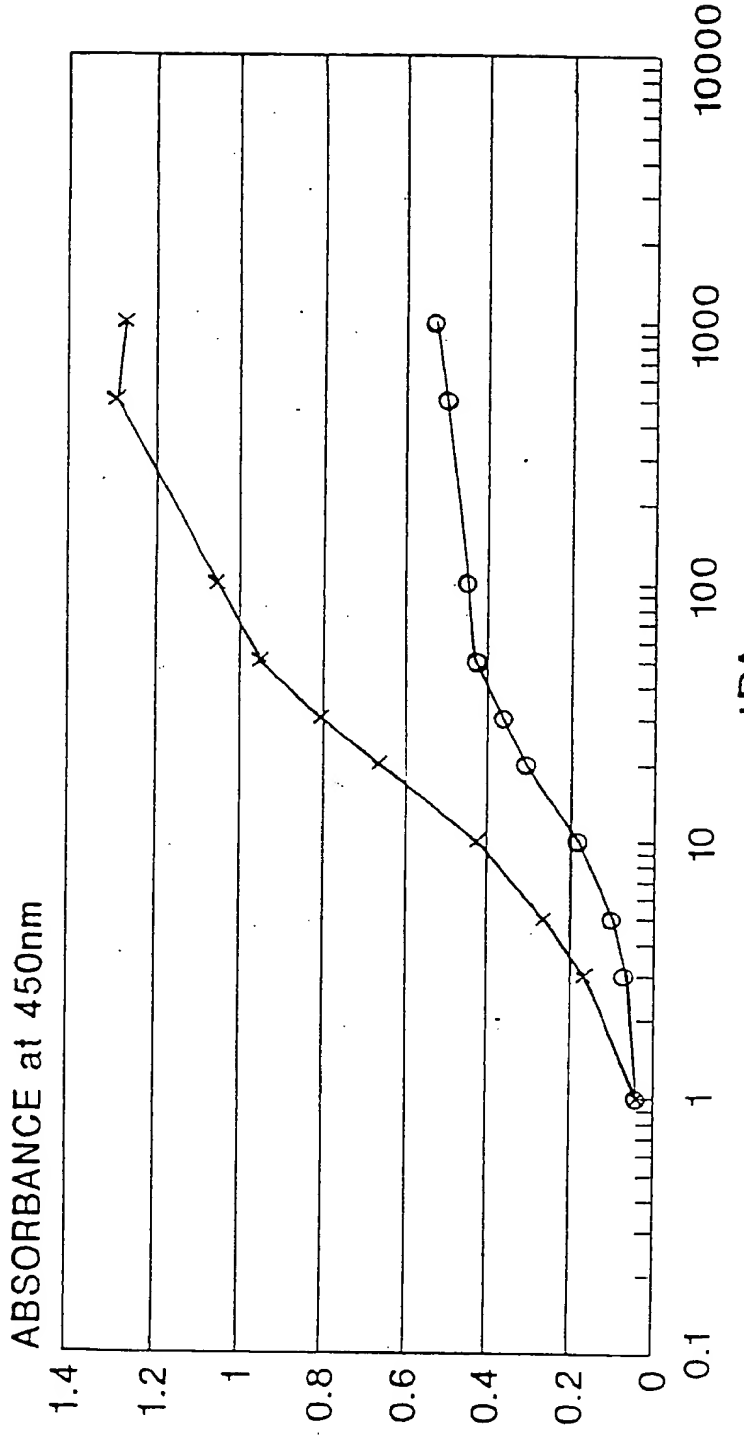
ABSORBANCE at 450nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.1
1
10
100
1000
10000
ng tPA
JMI-229 tPA
ACTILYSE

Fig. 3

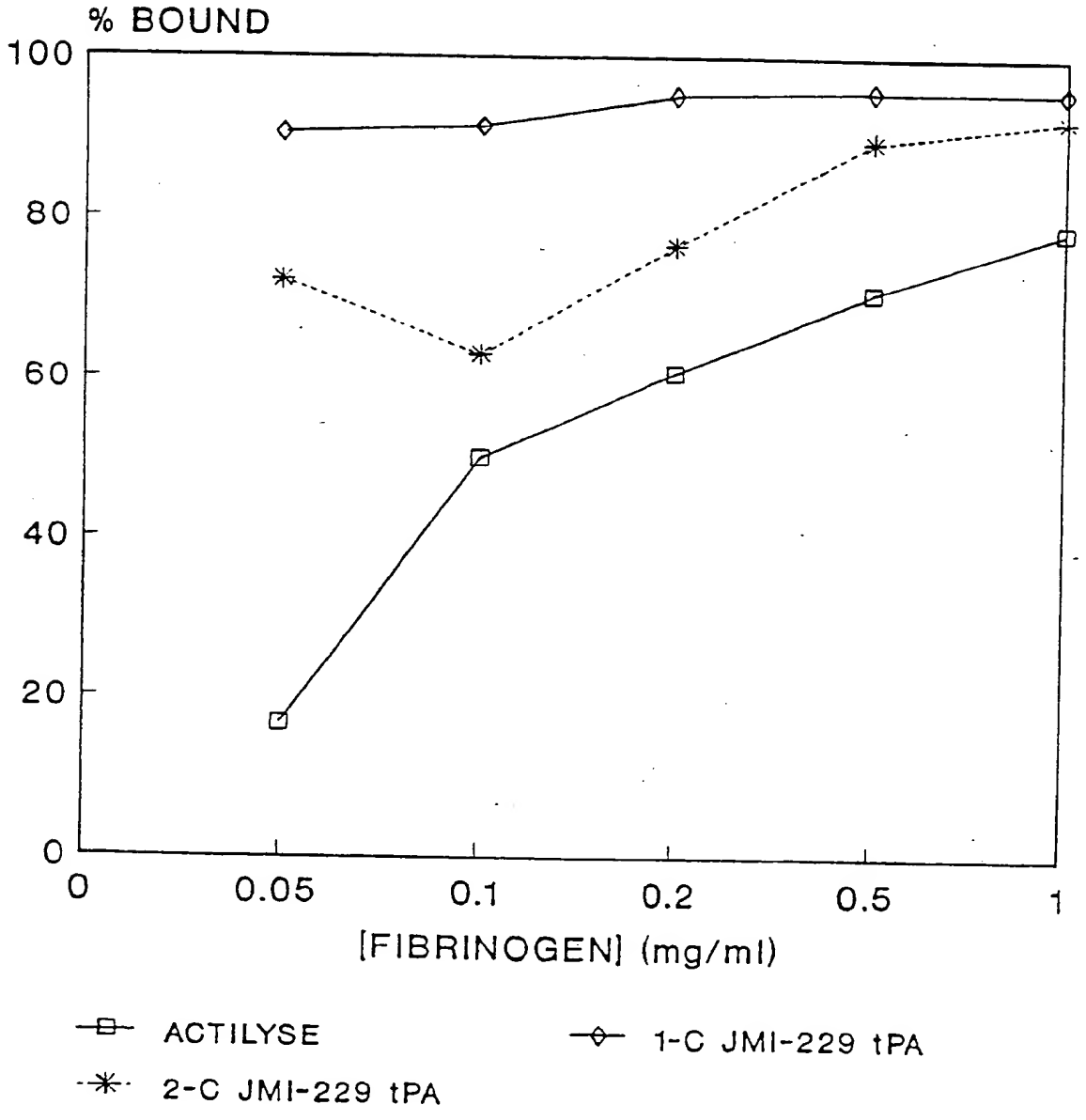
% BOUND
100
80
60
40
20
0
0
0.05
0.1
0.2
0.5
1
[FIBRINOGEN] (mg/ml)
ACTILYSE
1-C JMI-229 tPA
2-C JMI-229 tPA

Fig. 4

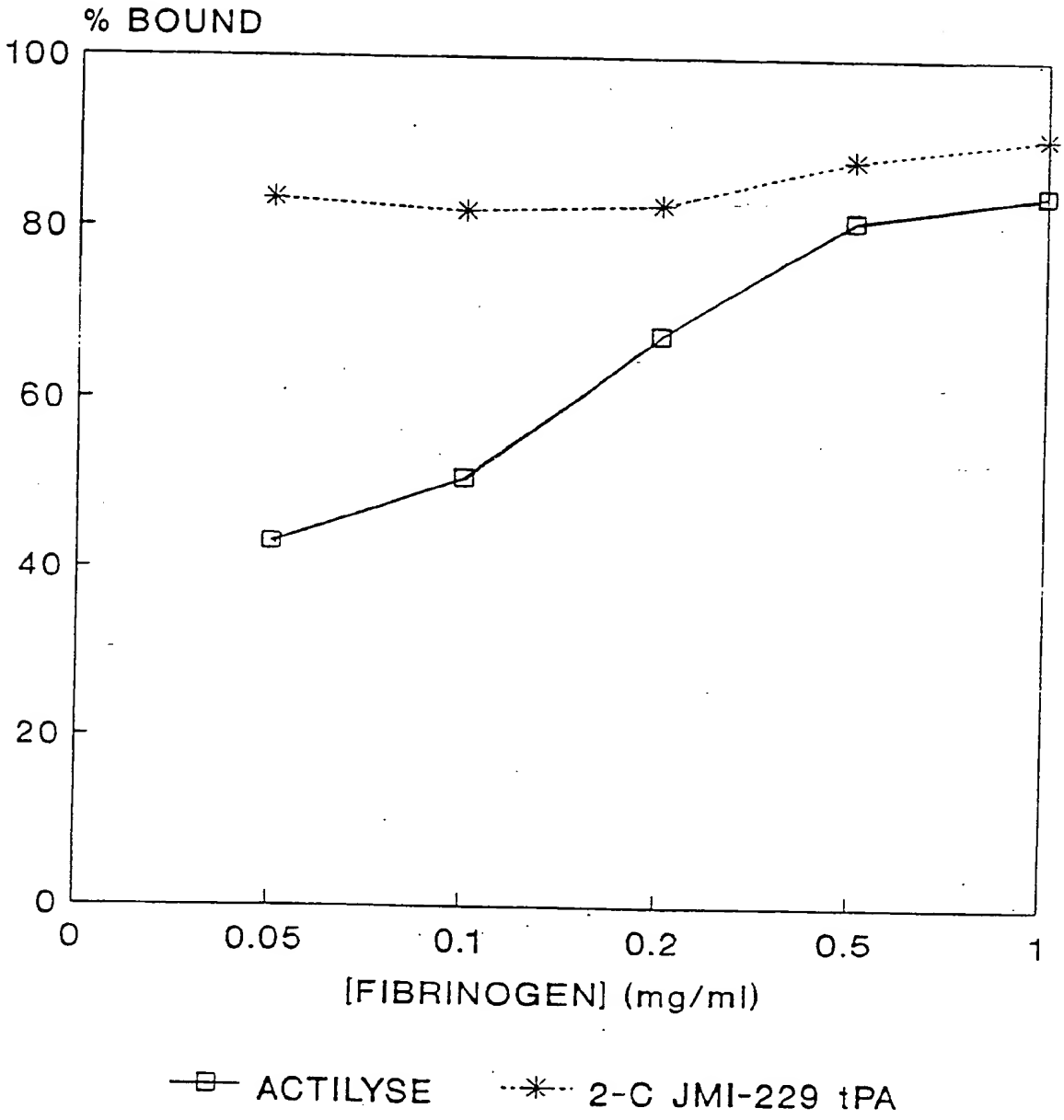
% BOUND
100
80
60
40
20
0
0
0.05
0.1
0.2
0.5
1
[FIBRINOGEN] (mg/ml)
ACTILYSE
2-C JMI-229 tPA

Fig. 5

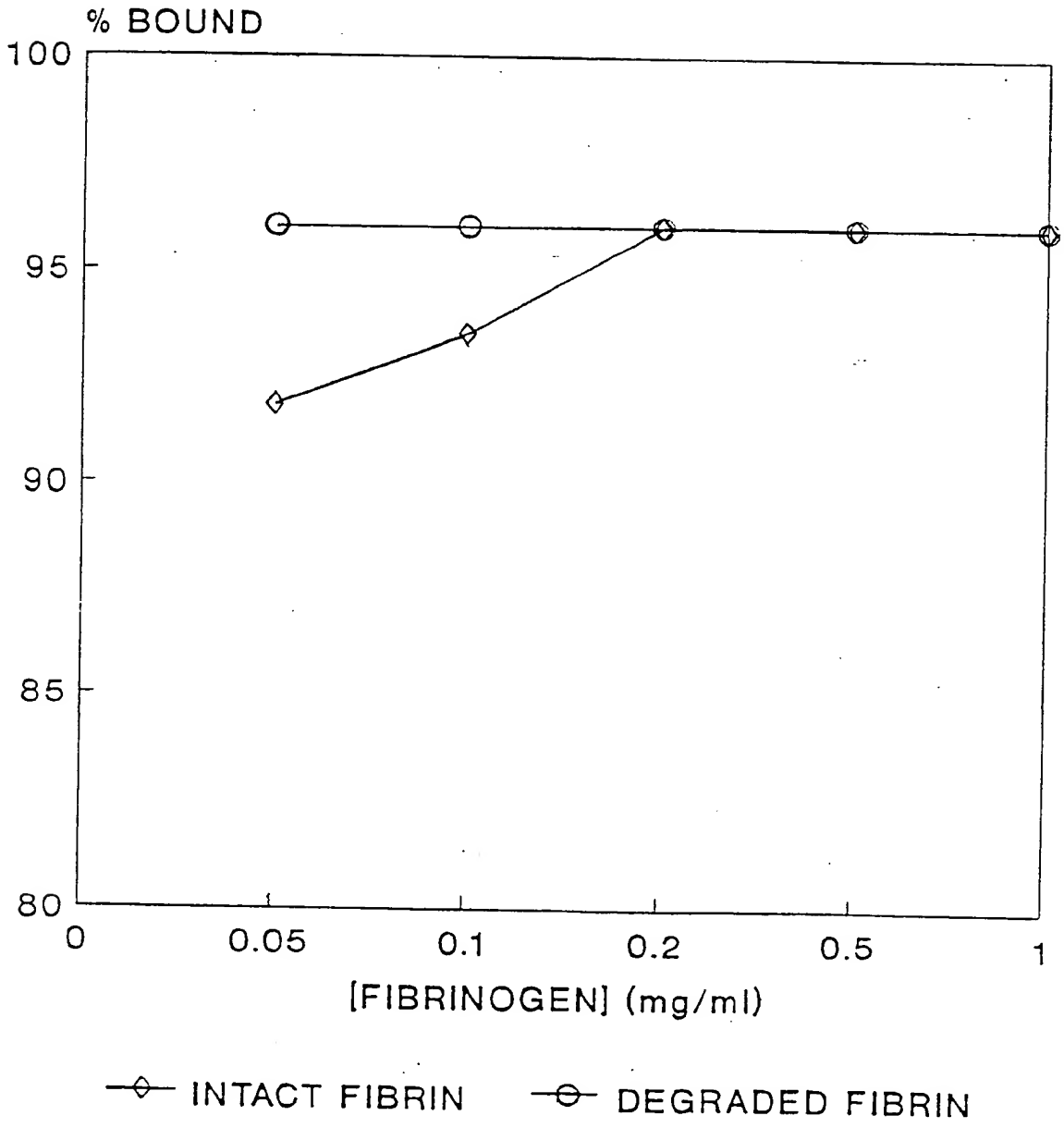
% BOUND
100
95
90
85
80
0
0.05
0.1
0.2
0.5
1
[FIBRINOGEN] (mg/ml)
INTACT FIBRIN
DEGRADED FIBRIN

Fig.6

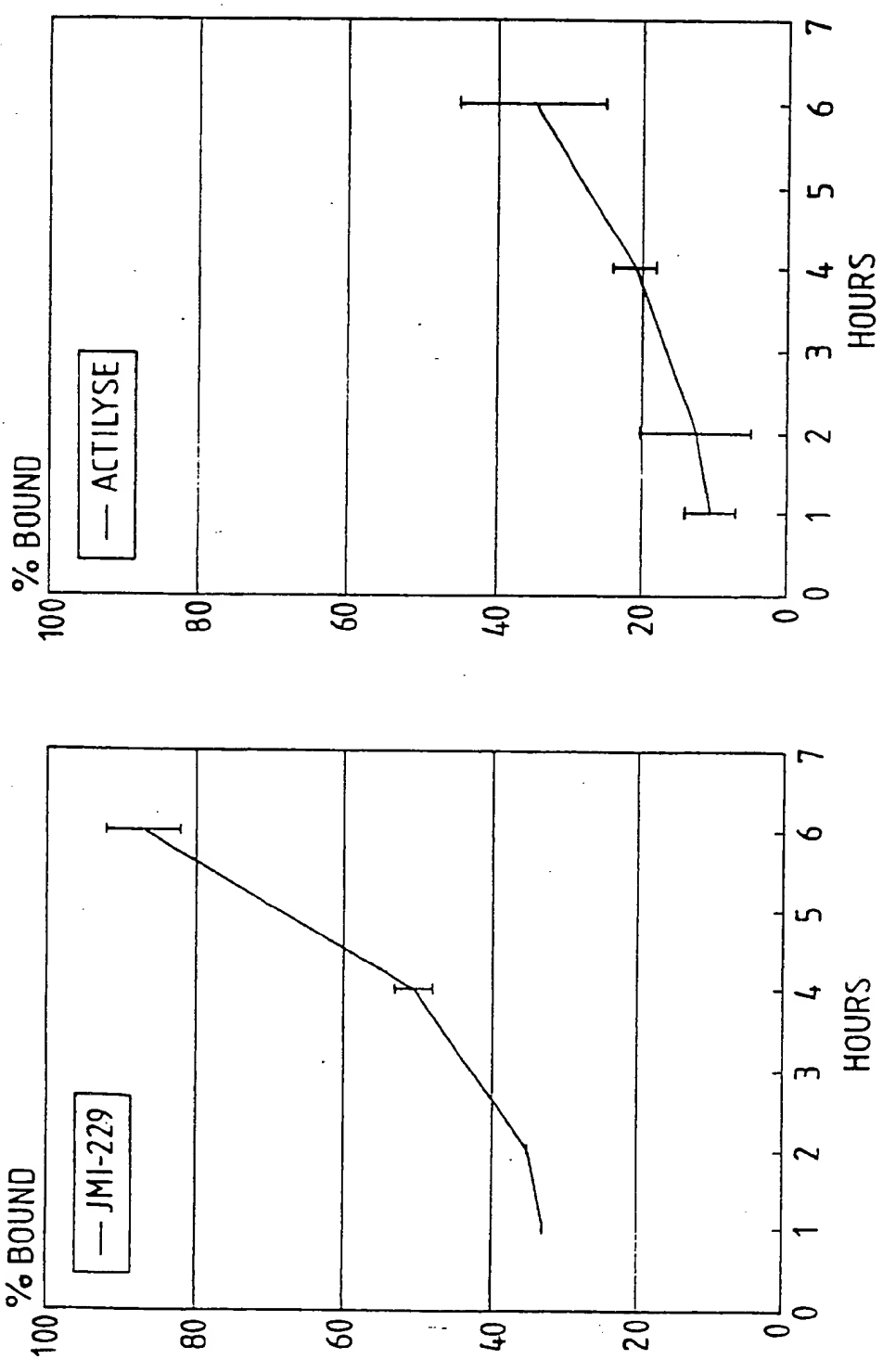
% BOUND
100
80
60
40
20
0
— ACTILYSE
0 1 2 3 4 5 6 7
HOURS
% BOUND
100
80
60
40
20
0
— JMI-229
0 1 2 3 4 5 6 7
HOURS

Fig. 7

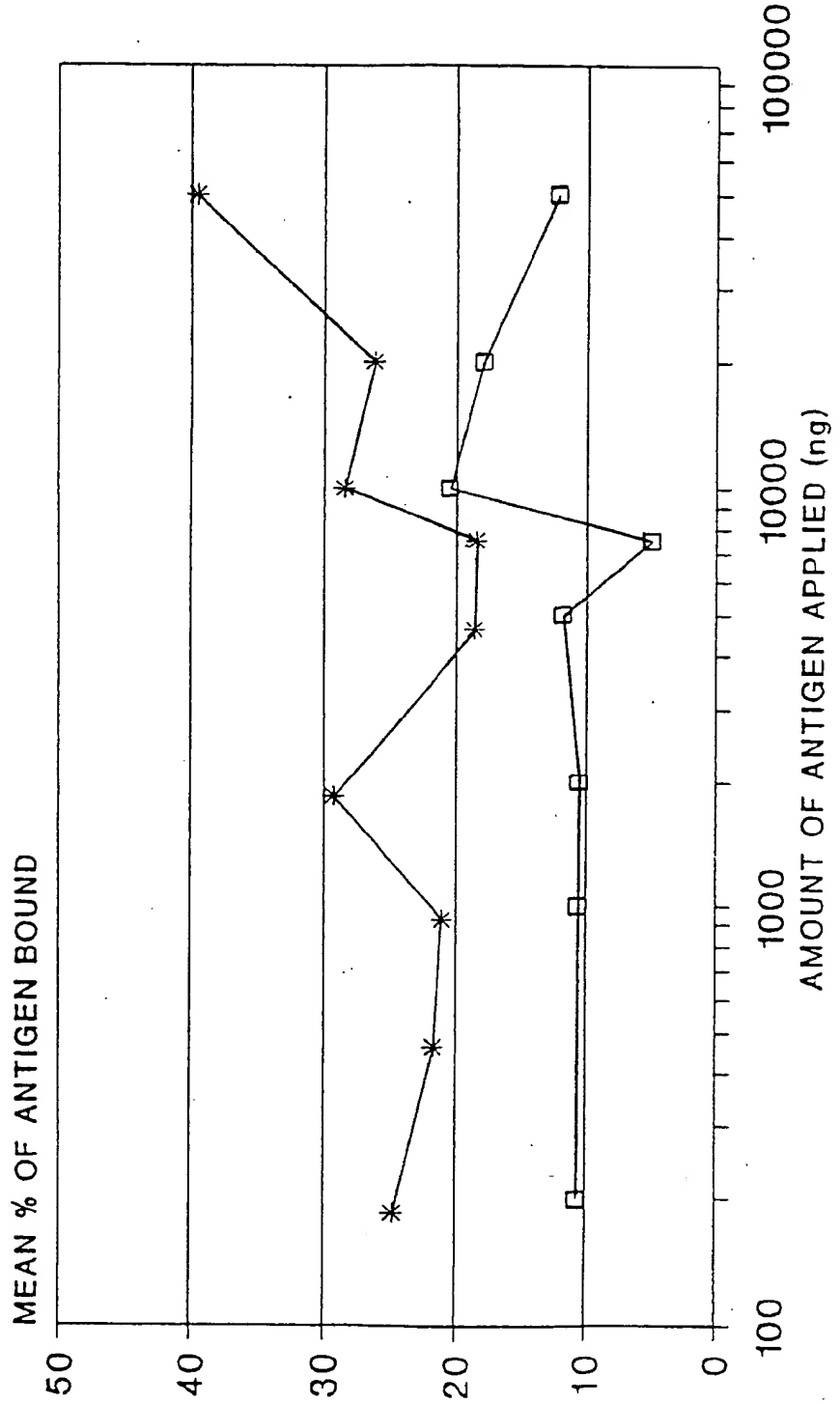
MEAN % OF ANTIGEN BOUND
50
40
30
20
10
0
100
1000
10000
100000
AMOUNT OF ANTIGEN APPLIED (ng)
JMI-229 tPA
ACTILYSE

Fig. 8

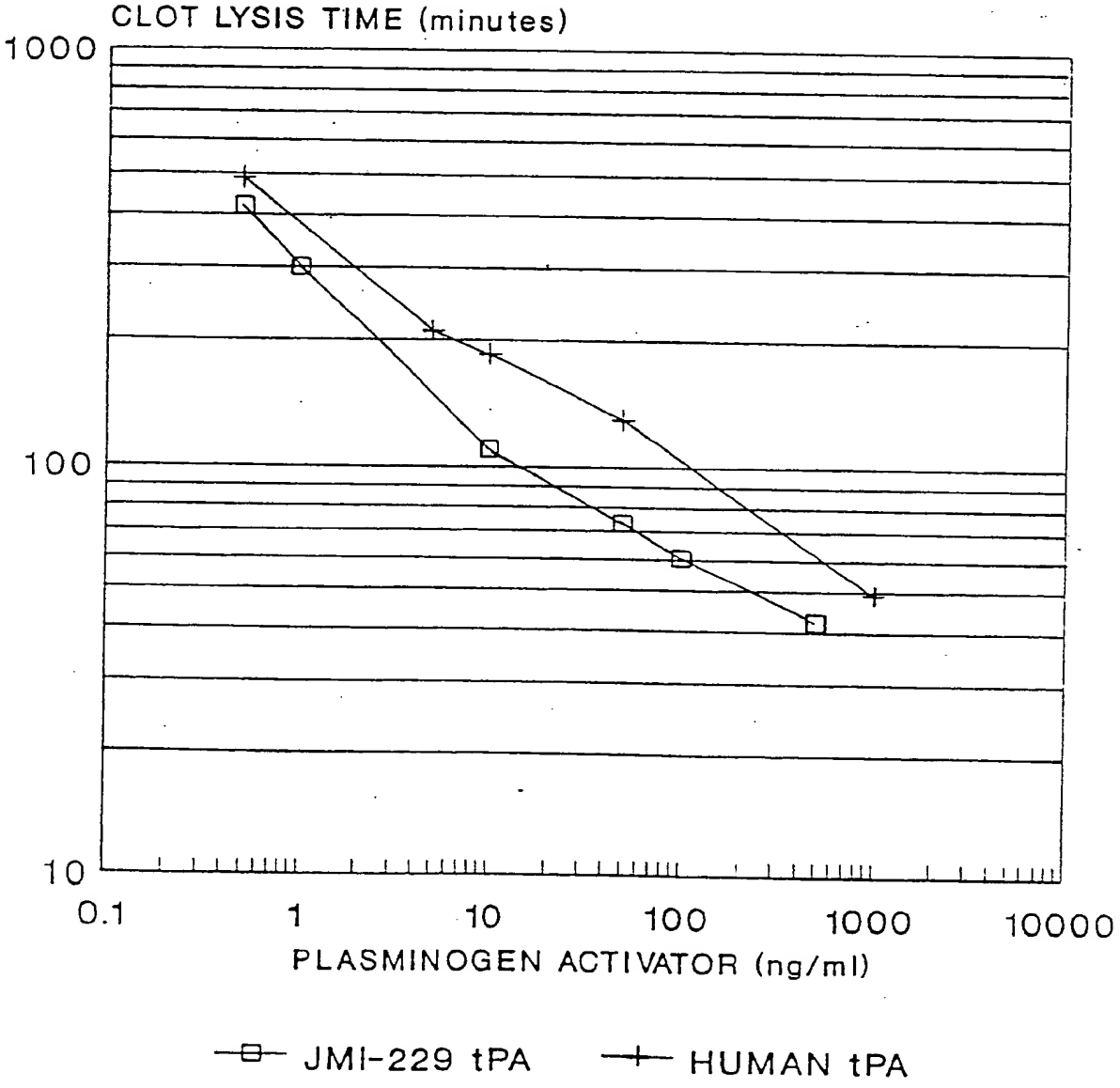
CLOT LYSIS TIME (minutes)
1000
100
10
0.1
1
10
100
1000
10000
PLASMINOGEN ACTIVATOR (ng/ml)
JMI-229 tPA
HUMAN tPA

Fig. 9

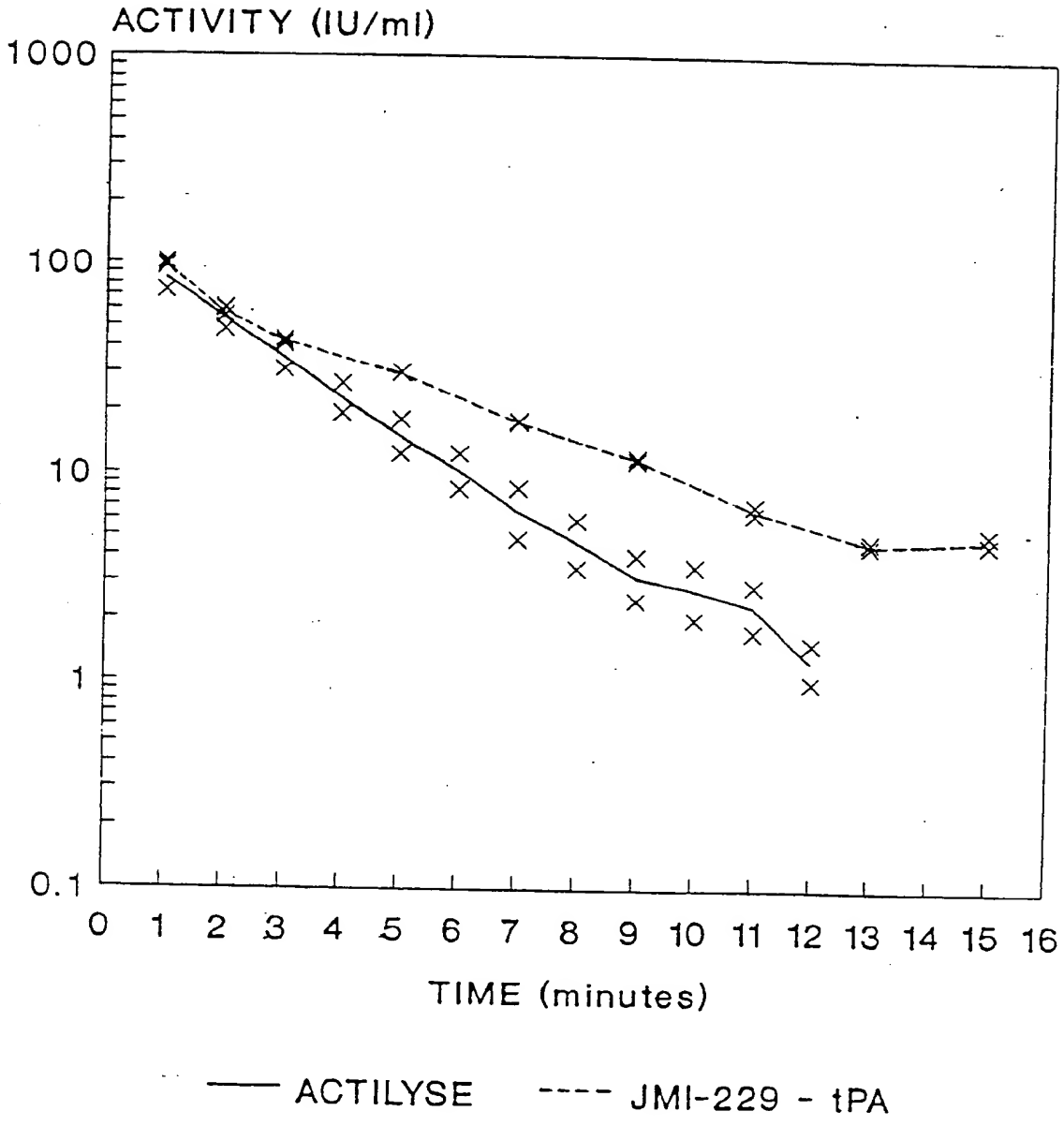
ACTIVITY (IU/ml)
1000
100
10
1
0.1
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
TIME (minutes)
ACTILYSE
JMI-229 - tPA

Fig. 10

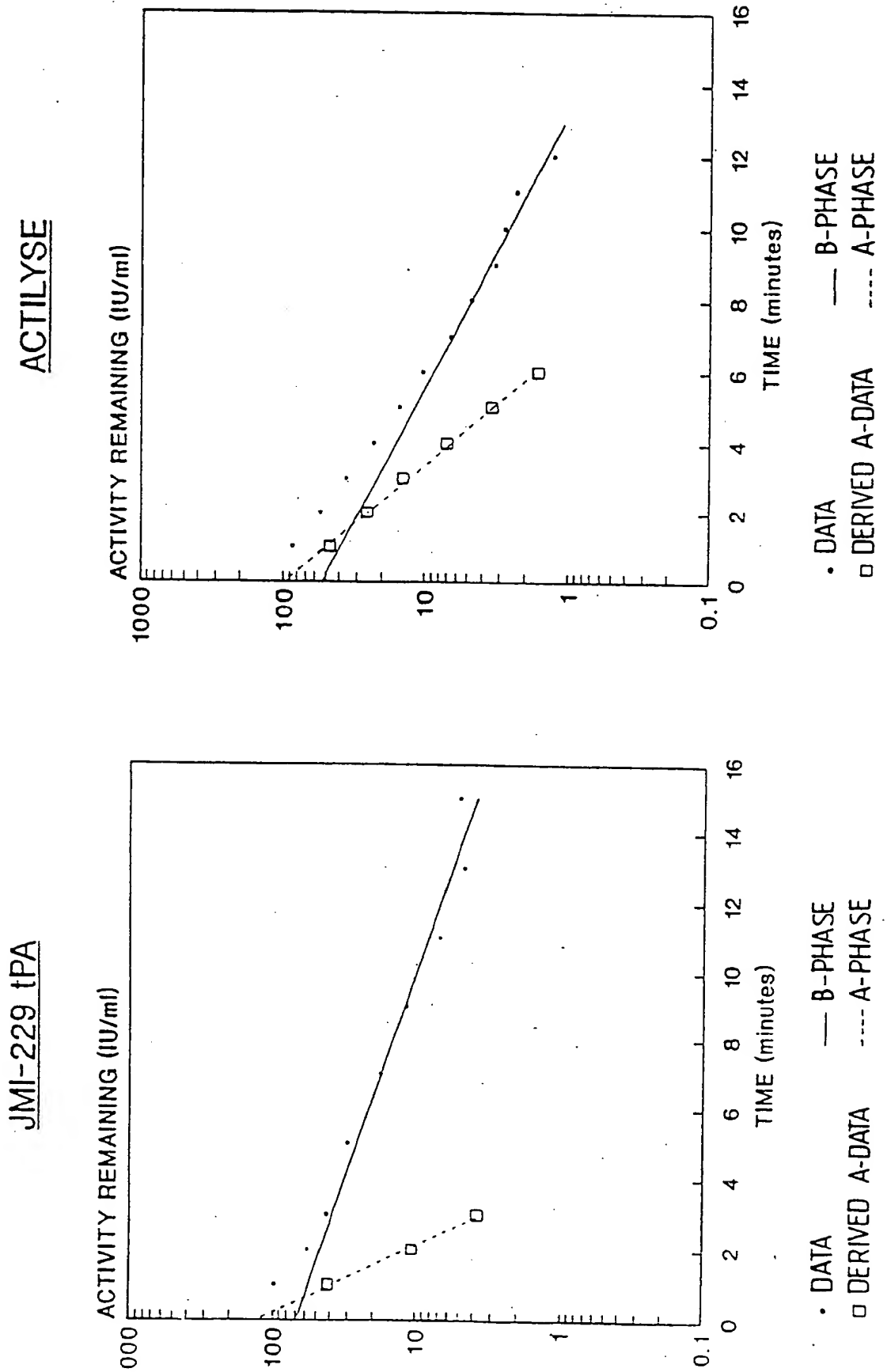
ACTILYSE
ACTIVITY REMAINING (IU/ml)
1000
100
10
1
0.1
0
2
4
6
8
10
12
14
16
TIME (minutes)
• DATA
□ DERIVED A-DATA
— B-PHASE
---- A-PHASE
JMI-229 tPA
ACTIVITY REMAINING (IU/ml)
1000
100
10
1
0.1
0
2
4
6
8
10
12
14
16
TIME (minutes)
• DATA
□ DERIVED A-DATA
— B-PHASE
---- A-PHASE

Fig. 11

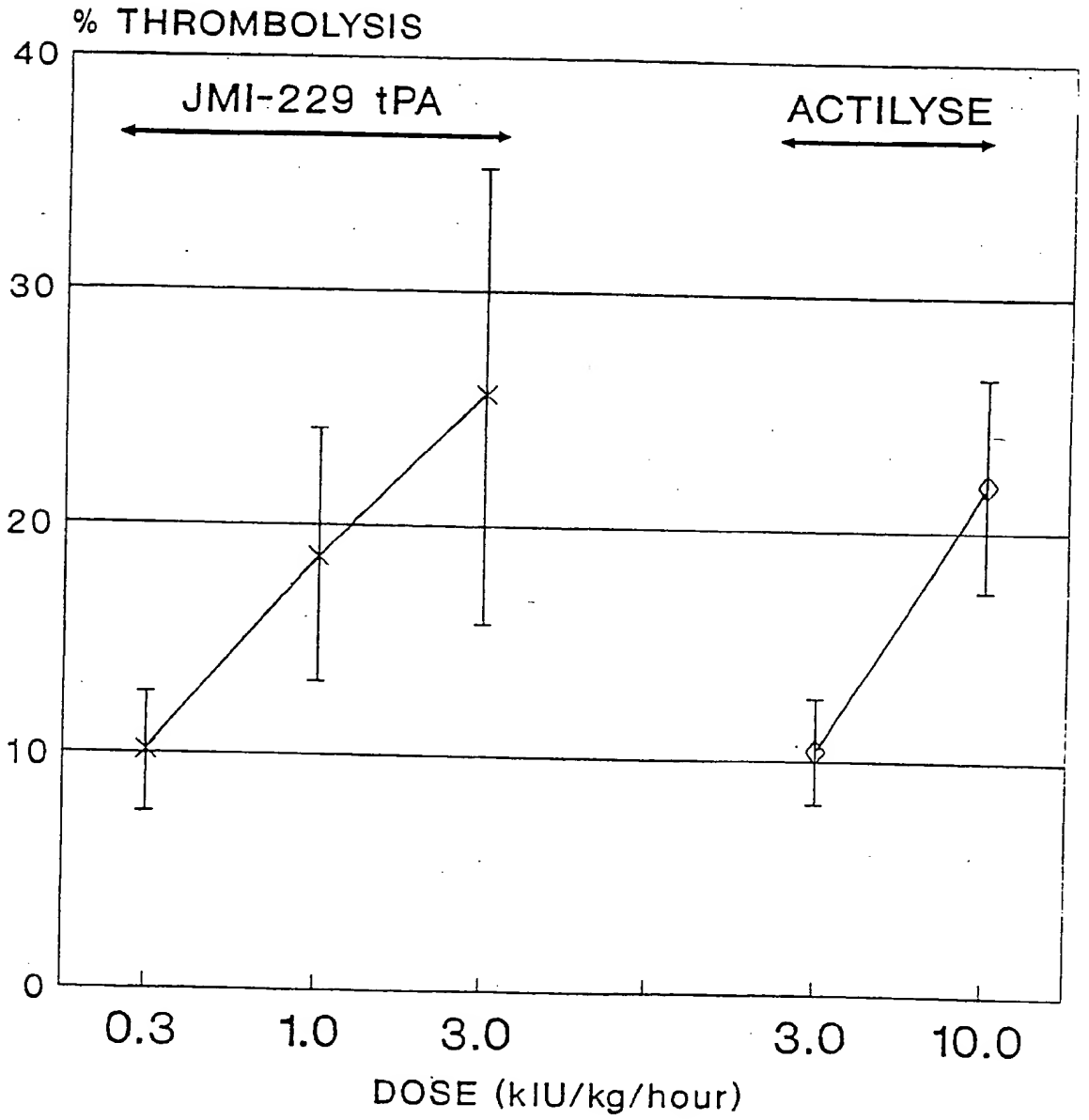
% THROMBOLYSIS
40
30
20
10
0
JMI-229 tPA
ACTILYSE
0.3
1.0
3.0
3.0
10.0
DOSE (kIU/kg/hour)

Fig. 12

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 91/00025

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: A 61 K 37/54, C 12 N 9/64,//C 12 N 15/58

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	A 61 K, C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	DNA, vol. 7, no. 10, December 1988, Mary Ann Liebert, Inc., Publishers, T. Ny et al.: "Cloning and characterization of a cDNA for rat tissue-type plasminogen activator", pages 671-677 see the whole article, especially page 674 --	1-7,9
A	WO, A, 8303101 (PUBLIC HEALTH LABORATORY SERVICE BOARD) 15 September 1983 see the whole document, especially claims and table 2 --	1-7,9
A	The Journal of Biological Chemistry, vol. 263, no. 3, 25 January 1988, The American Society for Biochemistry and Molecular Biology, Inc., (US), R.J. Rickles et al.: "Molecular cloning of complementary DNA to mouse tissue plasminogen activator mRNA and its expression during F9 teratocarcinoma ./.	1-7,9

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10th April 1991	17. 06. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. TORIBIO Maria TORIBIO

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages	Relevant to Claim No.
	cell differentiation", pages 1563-1569 see the whole article --	
A	WO, A, 8902917 (PORTON PRODUCTS) 6 April 1989 see the whole document --	1-7,9
A	US, A, 4661453 (MORRIS POLLARD) 28 April 1987 see the whole document cited in the application --	1-7,9
A	FR, A, 2593393 (THE WELLCOME FOUNDATION LTD) 31 July 1987 see the whole document --	1-7
A,P	The Journal of Biological Chemistry, vol. 265, no. 4, 5 February 1990, The American Society for Biochemistry and Molecular Biology, Inc., (US), P. Feng et al.: "The structure of the TATA-less rat tissue-type plasminogen activator gene", pages 2022-2027 see the whole article	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers 8 because they relate to subject matter not required to be searched by this Authority, namely:

Pls. see Rule 39.1(iv) - PCT:
Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. [ ] Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically: .

3. [ ] Claim numbers _____, because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9100025 SA 43650

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 07/05/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A- 8303101	15-09-83	AU-A-	1214688	08-09-88
		AU-B-	567491	26-11-87
		CA-A-	1213231	28-10-86
		DE-A-	3375491	03-03-88
		EP-A,B	0104194	27-01-88
		JP-T-	59500351	08-03-84
		SU-A-	1507204	07-09-89
		US-A-	4780412	25-10-88
WO-A- 8902917	06-04-89	AU-A-	2428888	18-04-89
		EP-A-	0395652	07-11-90
		US-A-	5002877	26-03-91
US-A- 4661453	28-04-87	None		
FR-A- 2593393	31-07-87	AT-B-	391812	10-12-90
		AU-B-	567236	12-11-87
		AU-A-	5796486	04-12-86
		AU-B-	569429	28-01-88
		AU-A-	5796586	04-12-86
		BE-A-	904830	27-11-86
		BE-A-	904831	27-11-86
		CH-A-	665356	13-05-88
		CH-A-	664495	15-03-88
		DE-A,C	3617752	04-12-86
		DE-A,C	3617753	04-12-86
		FR-A,B	2583984	02-01-87
		GB-A,B	2176702	07-01-87
		GB-A,B	2176703	07-01-87
		JP-A-	62026233	04-02-87
		JP-A-	62026234	04-02-87
		JP-B-	63038327	29-07-88
		LU-A-	86444	05-12-86
		LU-A-	86445	05-12-86
		NL-A-	8601354	16-12-86
		NL-A-	8601355	16-12-86
		SE-B-	462893	17-09-90
		SE-A-	8602404	29-11-86
		SE-B-	462016	30-04-90

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9100025 SA 43650

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 07/05/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
FR-A- 2593393		SE-A- 8602405	29-11-86
		US-A- 4968617	06-11-90
		US-A- 4929444	29-05-90